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THE INTERACTION OF MONOFLUOROFUMARATE WITH ADENYLOSUCCINATE LYASE

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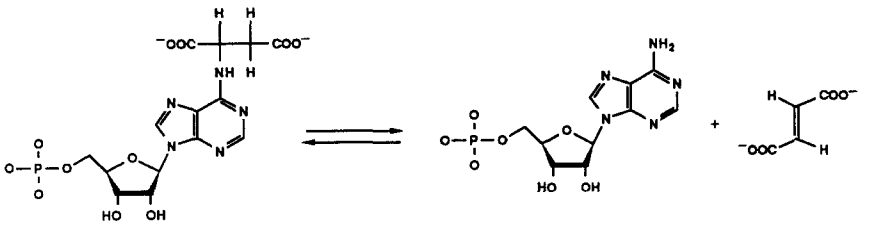
Monofluorofumarate was tested as an alternate substrate and inhibitor for adenylosuccinate lyase. Monofluorofumarate was found to be a slow reacting substrate when either AMP or AICAR (5-aminoimidazole 4-carboxamide ribonucleotide) were used as substrate acceptor molecules at pH 7.5. There was no indication that monofluorofumarate could induce the inactivation of adenylosuccinate lyase. The initial reaction product when monofluorofumarate was incubated with AMP in the presence of adenylosuccinate lyase has been determined to be 2-fluoro-adenylosuccinate. This molecule lost HF spontaneously, and the subsequent intermediate was rapidly hydrolyzed to oxalacetate and AMP. A similar reaction scheme was also observed when AICAR was utilized as a cosubstrate with monofluorofumarate. The initial reaction rate when 1.0 mM monofluorofumarate and 1.0 mM AMP were used as substrates with adenylosuccinate lyase was only 1.4% of the rate when 1.0 mM fumarate was used. AICAR (1.0 mM) was found to react with monofluorofumarate at 8.9% of the rate that it reacts with fumarate.

KEY WORDS: Adenylosuccinate lyase, monofluorofumarate.

INTRODUCTION

Adenylosuccinate lyase catalyzes the following two reactions during the biosynthesis of AMP:



adenylosuccinate AMP fumarate (1)

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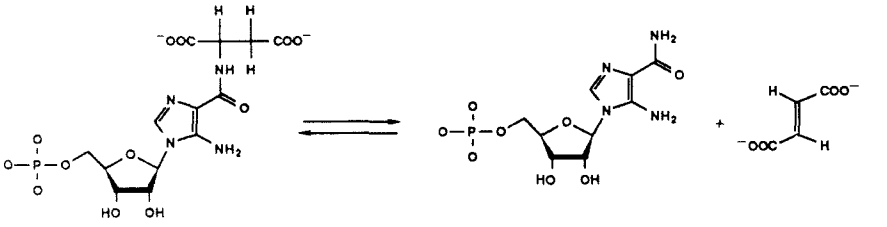
Abbreviations: AICAR 5-aminoimidazole 4-carboxamide ribonucleotide; SAICAR, 4-(N-succino)-5-aminoimidazole-4-carboxamide ribonucleotide.

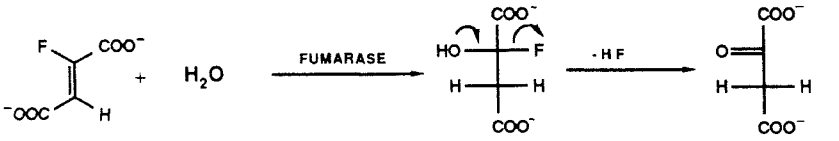


SAICAR AICAR fumarate (2)

Both of these reactions involve the α,β-elimination of a nitrogen nucleophile from a substituted aspartate precursor. The reactions are freely reversible. Porter *et al.* have shown that the nitro analog of adenylosuccinate, N^6-(*L*-1-carboxy-2-nitroethyl)-AMP, is a potent inhibitor of adenylosuccinate lyase. These studies strongly suggest that a carbanion is an intermediate in these reactions.[1]

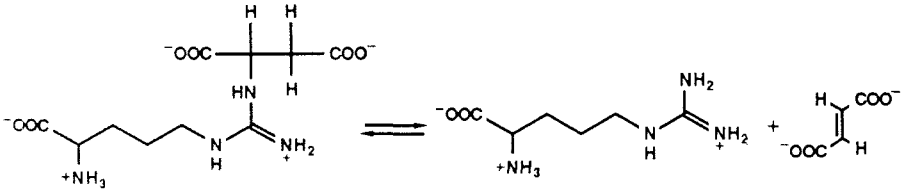
Casey *et al.* have enzymatically synthesized *threo*-3-fluoro-adenylosuccinate and *threo*-3-fluoro-SAICAR and tested these compounds as inhibitors for the reactions catalyzed by adenylosuccinate lyase. In 20 mM Hepes buffer, pH 7.4, the K_i values for *threo*-3-fluoro-SAICAR and *threo*-3-fluoro-adenylosuccinate were found to be 0.03 and 0.015 μM, respectively, for the competitive inhibition of the conversion of adenylosuccinate to AMP and fumarate.

Monofluorofumarate is an attractive analog for fumarate in enzymatic reactions.[3,4] For example, monofluorofumarate is hydrated by fumarase 4 times faster than is fumarate.[3] The addition of water occurs almost exclusively at the fluorinated carbon to form 2-fluoro-malate (equation 3). HF is spontaneously lost and oxalacetate is ultimately formed.[3,5] These results are also consistent with a carbonionic reaction intermediate.[6]



(3)

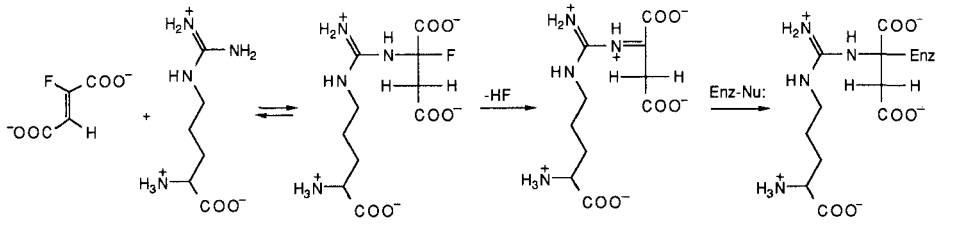
Monofluorofumarate has also been utilized as a substrate in the reaction catalyzed by argininosuccinate lyase.[4] This enzyme catalyzes the reversible cleavage of argininosuccinate to arginine and fumarate (equation 4).



argininosuccinate arginine fumarate (4)

When monofluorofumarate is used as a substrate analog for fumarate the guanidino nitrogen of arginine adds to the carbon bonded directly to the fluorine. The subse-

quent loss of HF results in the formation of a good Michael-type acceptor, which is then attacked by a nucleophile in the vicinity of the active site. The covalent-enzyme adduct is completely inactive. The rate of inactivation at saturating monofluorofumarate is 13 min^{-1}. However, the enzyme-inhibitor complex is reversible since full activity of argininosuccinate lyase could be recovered by removal of excess reagent. These reactions are summarized in Scheme I.



SCHEME I

Since the chemical and reaction mechanisms of adenylosuccinate lyase and argininosuccinate lyase are so similar, it was felt that monofluorofumarate would have a high probability for being a potent inactivator or inhibitor of the reaction catalyzed by adenylosuccinate lyase. In this paper, the reaction pathway for the reaction of monofluorofumarate with adenylosuccinate lyase has been established using NMR, HPLC, and UV absorption spectroscopy. The analog was found to be a substrate, but did not inhibit nor inactivate the enzymatic reaction in a manner similar to the previously obtained results with argininosuccinate lyase. The product of the reaction and possible reaction pathways are discussed.

MATERIALS AND METHODS

Adenylosuccinate lyase, AMP, AICAR, NADH, lactate dehydrogenase and malate dehydrogenase were purchased from Sigma Chemical Co. Monofluorofumarate was synthesized according to the procedure of Raasch *et al.*[7] starting from 1,1,2-trichloro-2,3,3-trifluorocyclobutane (PCP Research Chemicals). Proton NMR spectra were obtained with a Varian XL-200 spectrophotometer operating at a frequency of 200 MHz. Typical acquisition parameters were 2900 Hz sweep width, 5.64 s acquisition time, and 5.8 μsec pulse width. ^{19}F NMR spectra were obtained with a Varian XL-400 spectrophotometer operating at a frequency of 376 MHz. Typical acquisition parameters were 50000 Hz sweep width, 0.6 s acquisition time, 4 s delay time, and 5 μsec pulse width. Enzymatic assays for product and absorption spectra were measured with a Gilford 2600 UV-vis spectrophotometer and a Hewlett-Packard 7225A plotter.

Monofluorofumarate and AMP

The time course for the change of the absorption spectrum upon incubation of AMP, fluorofumarate and adenylosuccinate lyase was measured between 230 nm and 330 nm. Monofluorofumarate (1.0 mM), 0.5 mM AMP, and 100 mM Hepes buffer (pH 7.5) were incubated in a microcuvette with a 2 mm path length. The reaction was initiated by adding 1.5 units of enzyme. The proton and fluorine NMR spectra of the

reaction products were obtained by incubating a 10 mL solution of 50 mM phosphate buffer (pH 7.0), 5.0 mM AMP, 10 mM monofluorofumarate, and 30 units of adenylosuccinate lyase. After 48 hours the reaction was quenched by passage through an Amicon PM30 ultrafiltration membrane. After removal of all the H_2O by rotary evaporation, the products were dissolved in D_2O. This procedure was repeated. The proton and ^{19}F NMR spectra were then obtained.

The time course for the formation of intermediates and reaction products was obtained by incubating a 100 mL solution of 50 mM phosphate buffer (pH 7.0), 10 mM AMP, 10 mM monofluorofumarate, and 30 units of adenylosuccinate lyase at 25 C. Aliquots were removed after 4, 12, 26, 49, and 72 hours and the reaction was quenched by passage through an Amicon PM30 ultrafiltration membrane. Proton NMR spectra were taken in D_2O as described above. The concentrations of monofluorofumarate and the reaction intermediate were determined by comparison of the peak integrations. The concentration of oxalacetate and pyruvate were measured enzymatically. For the oxalacetate assay, 0.06 mL aliquots were removed at various times and mixed with 100 mM Hepes buffer (pH 7.5) and 0.2 mM NADH in a final volume of 3 mL. Malate dehydrogenase (0.1 units) was added and the change in the concentration of NADH was measured at 340 nm. For the pyruvate assay, 0.1 units of lactate dehydrogenase was utilized instead of malate dehydrogenase. The absorbance changes for the formation of the intermediate were followed at 308 nm.

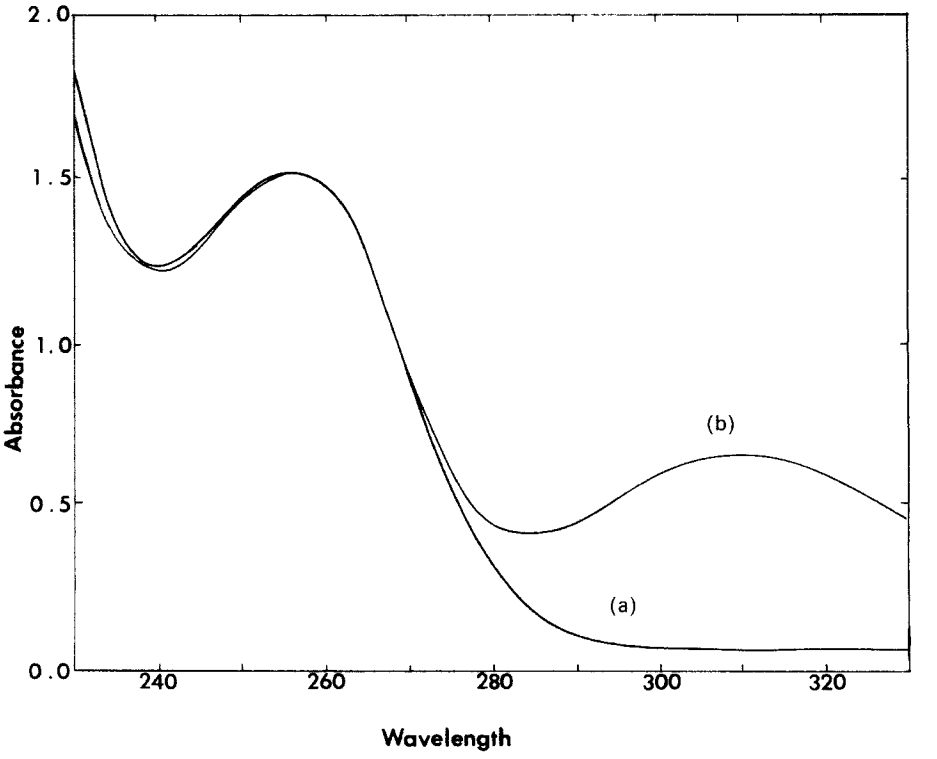


FIGURE 1 UV absorption spectra for the products and substrates before and after the incubation of adenylosuccinate lyase with AMP and fluorofumarate. (A) Before the addition of enzyme. (B) 24 hours after the addition of enzyme. Additional details are given in the text.

Monofluorofumarate and AICAR

The changes in the absorption spectrum upon incubation of fluorofumarate, AICAR and adenylosuccinate were measured between 230 nm and 330 nm. Monofluorofumarate (1.0 mM), 0.5 mM AICAR, and 100 mM Hepes buffer (pH 7.5) were incubated in a microcuvette with a 2 mm path length and the reaction was initiated by adding 0.5 units of enzyme.

The time course for the formation of products and intermediates was obtained by incubating 30 mL of 50 mM phosphate buffer (pH 7.0), 5.0 mM AICAR, 5.0 mM monofluorofumarate, and 15 units of adenylosuccinate lyase at 25°C. The change in the concentration of oxalacetate and pyruvate and the absorbance change at 306 nm were measured as described above. The concentration of monofluorofumarate and the intermediate was also analyzed by proton NMR spectroscopy in D_2O. The ^{19}F NMR spectrum was obtained 48 hours after the initiation of the reaction.

Relative Velocities

The relative rates for the reaction catalyzed by adenylosuccinate lyase with either fumarate or fluorofumarate were obtained under identical conditions. Each reaction solution contained 100 mM Hepes buffer (pH 7.5), 1.0 mM fumarate or monofluorofumarate, and 1.0 mM AMP or AICAR in 3 mL solution. Reactions were started by the addition of 0.01 units of enzyme when fumarate was a substrate. The rate of formation of adenylosuccinate, SAICAR, and the intermediate from monofluorofumarate and AICAR were detected by separation of the reaction products with HPLC. Aliquots were loaded onto a Whatman SAX anion exchange column and eluted with 0.25 M KH_2PO_4 elution buffer at 1.0 mL/min. Formation of the intermediate from monofluorofumarate and AMP was monitored spectrophotometrically at 308 nm.

RESULTS AND DISCUSSION

Reaction of Monofluorofumarate with AMP

The time course for the reaction between monofluorofumarate and AMP as catalyzed by adenylosuccinate lyase was monitored by NMR and UV spectroscopy. The UV spectrum of adenylosuccinate has an absorbance maximum at 267 nm at pH 7.5. The standard assays for enzymatic activity of adenylosuccinate lyase are usually monitored at 280 nm where the maximum difference in the extinction coefficient between adenylosuccinate and AMP occurs.[8] The difference in the molar extinction coefficient is 10,700. When adenylosuccinate lyase is added to a solution of AMP and monofluorofumarate, the absorbance maximum at 259 nm does not change as the reaction proceeds but a new absorbance maximum at 308 nm appears with time (Figure 1).

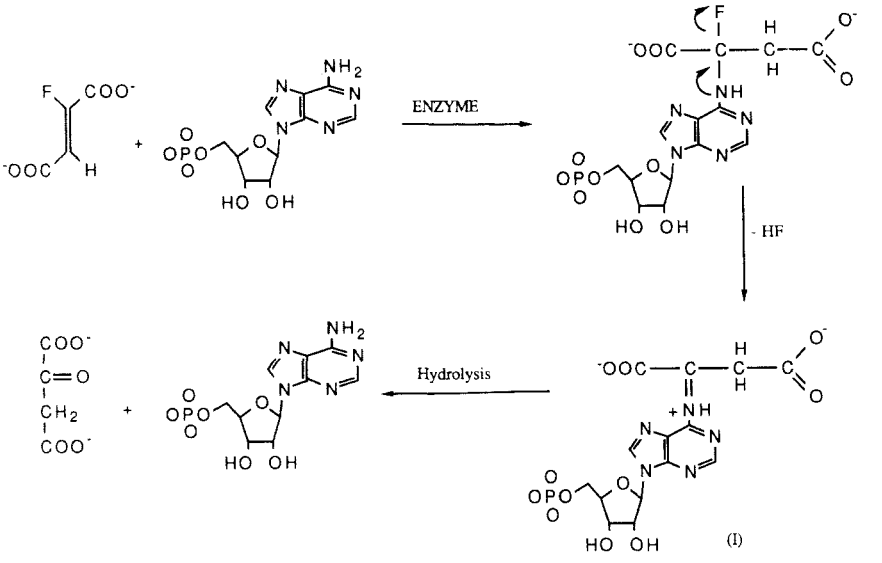
The change in the chemical environment of the fluorine atom in monofluorfumarate was monitored with ^{19}F NMR. The ^{19}F NMR spectrum of monofluorofumarate consists of a single doublet ($^3J_{HF}$ = 35 Hz) at 116 ppm upfield from $CFCl_3$.[4] After incubating monofluorofumarate, excess AMP and adenylosuccinate lyase for 48 hours the doublet for monofluorofumarate disappears and is replaced by a singlet at 125 ppm. The new singlet has the same chemical shift as F^-.

The product of the reaction was also analyzed by ^1H NMR spectroscopy at

200 MHz. As the reaction progressed very little change was noted for the protons on carbons 2′, 3′, 4′, and 5′ of the ribose ring. Small shifts were observed for the anomeric hydrogen and the two hydrogens at C-2 and C-8 of the adenine ring. A new resonance of an intermediate appeared at 5.42 ppm.

The formation of oxalacetate and pyruvate with time was measured enzymatically with malate dehydrogenase and lactate dehydrogenase respectively. After 48 hours of incubation with adenylosuccinate lyase and AMP a significant fraction of the fluorofumarate was converted to pyruvate and oxalacetate. Shown in Figure 2 is the time course for the condensation of fluorofumarate and AMP as catalyzed by adenylosuccinate lyase. Plotted in this figure are the disappearance of fluorofumarate, the formation of the intermediate, oxalacetate, pyruvate, and the change in absorbance at 308 nm.

The proposed scheme for the reaction of monofluorofumarate and AMP as catalyzed by adenylosuccinate lyase is shown in Scheme II. The amino group attached



SCHEME II

to C-1 of the adenine moiety adds to the carbon of fluorofumarate that is bonded directly to the fluorine and thus forms 2-fluoro-adenylosuccinate. This unstable molecule rapidly loses HF to form an unsaturated intermediate (I). Hydrolysis of the intermediate reforms the AMP and produces oxalacetate. The appearance of pyruvate results either from the nonenzymatic decarboxylation of oxalacetate or the decarboxylation of the intermediate prior to hydrolysis.

The initial formation of 2-fluoro-adenylosuccinate is supported by the observation that the F^- is lost as the reaction proceeds. If the condensation reaction had resulted in the formation of 3-fluoro-adenylosuccinate the fluorine would not have been labile. Moreover, the 3-fluoro analogs of adenylosuccinate synthesized by Casey *et al.* do not lose F^- in the presence of adenylosuccinate lyase and are not substrates.[2] After F^- is lost a Schiff's base is formed that is susceptible to either hydrolysis or decarboxylation. Since a lag is observed in the formation of pyruvate it is assumed that the

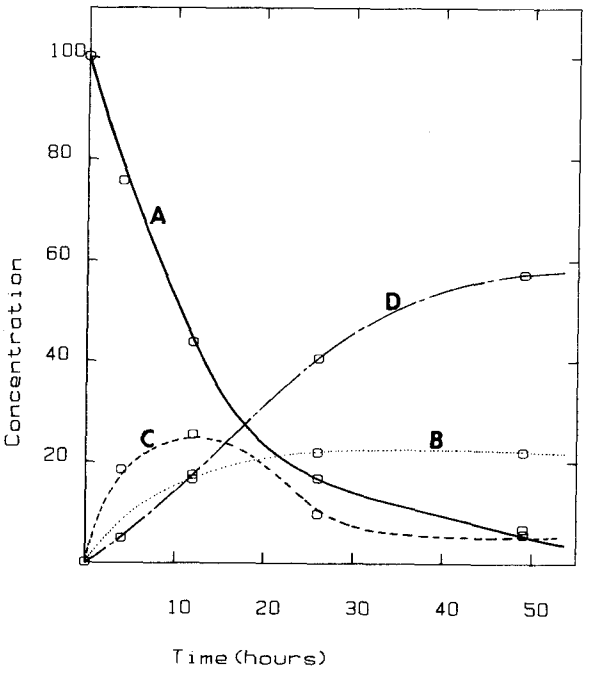
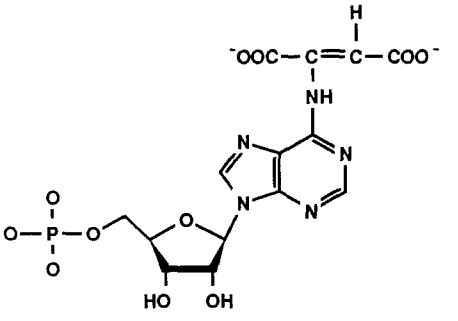
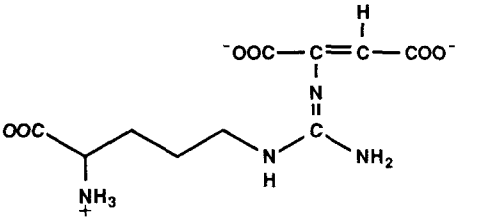


FIGURE 4 Time course for the reaction between AMP and fluorofumarate as catalyzed by argininosuccinate lyase. (A) Fluorofumarate. (B) Intermediate. (C) Oxalacetate (D) Pyruvate. Additional details are given in the text.

pathway where hydrolysis precedes carboxylation is predominant. An alternative mechanism can also be postulated. In this mechanism the appearance of the absorbance maximum at 308 nm is due to the formation of an eneamine (II) intermediate. This eneamine could be formed either by the tautomerization of the intermediate imine (I) or by the direct loss of fluoride and a proton from C-3 of 2-fluoro-adenylosuccinate. In the related reaction catalyzed by argininosuccinate lyase the most stable product is a conjugated eneamine (III) of similar structure.[4] This compound has an absorbance maximum at 299 nm.



(II)



(III)

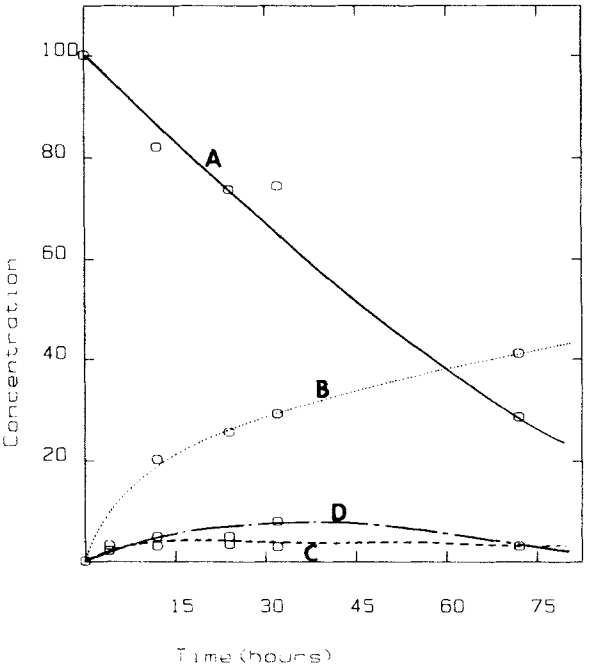


FIGURE 3 Time course for the reaction between AICAR and fluorofumarate as catalyzed by argininosuccinate lyase. (A) Fluorofumarate. (B) Intermediate. (C) Oxalacetate (D) Pyruvate. Additional details are given in the text.

Reaction of Monofluorofumarate with AICAR

A similar reaction mechanism is observed when fluorofumarate and AICAR are incubated with adenylosuccinate. Fluoride ion is produced quantitatively and a new absorbance maximum is observed at 306 nm. The time courses for these changes are summarized in Figure 3. A mechanism related to that shown in Scheme II can also be written for reaction of AICAR with fluorofumarate. This proposal is supported by the formation of F^-, oxalacetate, pyruvate and the absorbance maximum at 306 nm.

Relative Rates

The relative rates for the condensation of fumarate and fluorofumarate with ATP and AICAR were determined by HPLC and UV spectroscopy. At a concentration of 1.0 mM for all substrates it was observed that fumarate reacts 2.9 times faster with AICAR than with AMP. When fluorofumarate is utilized as a substrate analog the relative rate is 1.4 and 25% of the base rate with AMP and AICAR, respectively.

Inactivation of Adenylosuccinate Lyase

We could obtain no evidence to indicate that fluorofumarate could induce the inactivation of adenylosuccinate lyase. This observation is in contrast to the very rapid rate of inactivation observed previously with argininosuccinate lyase.[4] The

reason for this difference is not clear. However, this result could suggest that the postulated enzyme-nucleophile is not in the proper orientation to react rapidly enough with the putative intermediate. Alternatively, it could be argued that the Michael-type intermediates formed after the loss of F^- are not as chemically reactive as the one formed with argininosuccinate lyase.

Acknowledgements

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